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Gastrointestinal mixed adenoneuroendocrine carcinoma (MANEC): An immunohistochemistry study of 13 microsatellite stable cases

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ABSTRACT

Background: Mixed adenoneuroendocrine carcinoma (MANEC) is currently included in the category of neuroendocrine carcinomas but the therapeutically management is not yet defined.
Aims: To present the immunohistochemical (IHC) features of the epithelial mesenchymal transition (EMT) of MANEC.
Materials and methods: The clinicopathological features of 13 consecutive cases of MANEC (6 gastric and 7 colorectal) were correlated with the IHC expression of the biomarkers E-cadherin, β-catenin, N-cadherin, vimentin, maspin, CD44 and S100. In all of the cases open surgery was performed.
Results: All of the cases showed microsatellite stable status, expressed E-cadherin and membrane β-catenin in both components (neuroendocrine and adenocarcinoma) and were negative for N-cadherin, vimentin and S-100. The colorectal MANECs were negative for maspin. In gastric MANECs, maspin showed cytoplasm positivity in the neuroendocrine component and nuclear translocation in the adenocarcinoma cells. CD44 was positive in all of the cases, in both components. No tumor buddings were identified. Three of the 13 patients survived for at least 32 months, all of them showing lymphatic emboli but not lymph node metastases. Pure neuroendocrine lymph node metastases were seen in only four of the cases: one from stomach, two of the ascending colon and two cases of the upper rectum.
Conclusions: Gastrointestinal MANEC is a microsatellite stable tumor with nodular growth, which components might originate from a CD44-positive stem-like precursor cell. Lymph node status remains the most reliable prognostic parameter and agressivity seems to not be influenced by tumor budding degree or EMT-related features. The histologic aspect of metastatic component (neuroendocrine versus adenocarcinoma) should be included in the histopathological reports and might be used for establishing the proper-targeted therapy of MANEC.

1. Introduction

The neuroendocrine carcinomas (NEC) of the gastrointestinal tract are rare but highly aggressive tumors with high risk for lymph node metastases [1,2]. The mixed adenoneuroendocrine carcinoma (MANEC) or mixed neuroendocrine-non-neuroendocrine neoplasm (MiNEN) is defined by the World Health Organization (WHO) as being histologically composed by two intermingled components, adenocarcinoma and neuroendocrine component; each of them has to represent at least 30% of the tumor [1,3]. This term was introduced in the clinical practice in 2010 [1,4]; the first papers were reported in 2011. In the Medline database few than 100 studies have been published till August 2018 [5] and another 33 from August 2018 till October 2019.

MANECs can be diagnosed in the gallbladder, ampulla of Vater (25 cases reported till 2019), pancreas, liver, extrahepatic biliary tract (9 cases reported till 2019) and gastrointestinal tract [3–9]. Uncommon locations were reported for uterine cervix and five cases were reported in the urinary bladder [8,9]. However, they can occur in any other organs. Few than 40 MANECs of the stomach and 8 cases involving the cecum were reported till 2015 [7]. Between 2015–2019 the number of

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reported cases increased but most of them are case reports. A recent review counted a total number of 93 reported colorectal MANECs, in 14 case reports and 4 retrospective cohorts [5]. From them, 29 cases involved the proximal colon, 10 were located in the transverse colon, 25 in the distal colon and 12 in the rectum [5]. In another study, 40 cases of gastric MANECs were retrospectively evaluated [10]. As most of the studies were published as case reports, the histogenesis of MANEC is still unknown and the best targeted treatment strategy is not well defined [4,5,10,11].

In this paper we present a comprehensive immunoprofile of gastric and colorectal MANECs and, based on a case series and literature data, a hypothesis regarding histogenesis and aggressivity of these uncommon neoplasms. The epithelial mesenchymal transition (EMT), maspin subcellular localization and the CD44 expression of MANEC was firstly examined in literature and presented as an original contribution.

2. Materials and methods

We have retrospectively evaluated 13 consecutive cases of MANEC diagnosed in our department between July 2013 and June 2017. The agreement of the Ethical Committee of University of Medicine and Pharmacy of Tirgu MUres, Romania, was obtained for evaluation of the presented cases.

The clinicopathological parameters (Table 1) were correlated with the immunoprofile of the tumor cells. The diagnostic was based on the dual components (adenocarcinoma and neuroendocrine), which were identified in Hematoxylin Eosin, based on the current WHO criteria [1]. The two components were immunohistochemically (IHC) confirmed using the markers synaptophysin, chromogranin, neuron specific enolase (NSE), carcinoembryonic antigen (CEA), cytokeratin AE1/AE3, cytokeratin 7 and cytokeratin 20. The tumor grade was appreciated based on the Ki67 index in both components. The details about IHC markers are shown in Table 2. The tumor buds were quantified based on the current protocols used for colorectal carcinomas, using the IHC markers cytokeratin AE1/AE3 and maspin [12,13].

The microsatellite status was IHC assessed using the markers MLH-1, MSH-2, MSH-6 and PMS-2. Maspin was quantified as negative, cytoplasm only and mixed positive tumors (nucleus and cytoplasm). The EMT was analyzed using the IHC markers E-cadherin, β-catenin, N-cadherin and vimentin. CD44 was used to explore the stemness features of the tumor cells [13].

Those cases showing loss of E-cadherin and membrane to nuclear translocation of β-catenin were considered as presenting EMT. Positivity for N-cadherin and vimentin were also checked for identification of mesenchymal features. A cut-off of 10% was used to consider positive stains.

3. Results

In our university hospital there are about 60 gastric carcinomas and 150 colorectal carcinomas diagnosed every year. The 13 MANECs (7 colorectal and 6 from stomach) represented 2.5% from all of gastric carcinomas and 1.16% from all colorectal carcinomas diagnosed in our Department of Pathology. They were identified in both males and females with a median age of 63.55 ± 12.13 years (range 46–78 years).

From the 7 colorectal MANECs, 3 involved the proximal colon and 4 cases were identified in the rectum. The gastric tumors were equally distributed between the proximal stomach and antral region. All of the tumors were ulcerated and lymphatic invasion (with or without emboli) was present in all of the cases, independently from presence or absence of the lymph node metastases.

In all of the tumors, the neuroendocrine component was diffusely marked by synaptophysin and NSE and showed focal positivity for chromogranin and cytokeratin AE1/AE3. The monoclonal CEA was positive or negative (Table 3). Negativity for the neuroendocrine markers was used to identify the adenocarcinoma component (Fig. 1).

All of the cases presented a proficient mismatch repair proteins (MMR) status, showing positivity for MLH-1, MSH-2, MSH-6 and PMS-2 in both components (Table 3). They were considered as having microsatellite stable status (MSS).

In all of the cases, E-cadherin and β-catenin diffusely marked the tumor cells membrane in both components, without nuclear expression for β-catenin. No positivity for N-cadherin and vimentin was observed. In the neuroendocrine component, the CD44 expression was observed in all cases, with increasing percentage of the positive cells in the high-grade neuroendocrine component (Fig. 2).

Maspin was negative or its expression was present in the cytoplasm of the cells from the neuroendocrine component. The adenocarcinoma component showed negativity in colorectal MANECs and mixed maspin expression (cytoplasm and nucleus) in those localized in the stomach (Figs. 1 and 2).

Only 4 out of the 13 patients survived for at least 20 months, all of them being diagnosed in relatively early stages (Table 3): case 1 from cecum (stage pT4bN0), case 5 from upper rectum (stage pT1aN1, with pure neuroendocrine metastatic component), case 8 from gastro-esophageal junction (stage pT3N0, and case 10 from proximal stomach (stage pT2N0).

In one gastric (case 13) and two colorectal MANECs (cases 2 and 3) the lymph node metastatic cells showed pure neuroendocrine architecture. One patient (case 7, upper rectum) was lost from follow-up programme). In the other three gastric (cases 9, 11, 12) and two colorectal metastatic MANECs (cases 4 and 6), independently from the percentage of the two components and/or cytological degree of primary tumor, both adeno- and neuroendocrine components have been identified in the lymph nodes (Table 3).

4. Discussion

In our study, MANEC represented 2.5% from all of gastric carcinomas and 1.16% from all colorectal carcinomas diagnosed in the Department of Pathology. The percentage reported in literature confirm the rarity of this tumor and was shown to be about 3% from all carcinomas of the colon and rectum [14].

The first gastric carcinoma with dual components, exo-and endocrine, was described by Cordier in 1924 [15,16]. The immunoprofile of the tumor cells allowed then to classify the mixed adeno-neuroendocrine carcinomas in the following categories (Lewin's classification): MANEC (intermingled components, each of them represented at least 30%), collision tumors (the two components show a clear delimitation), and amphicrine-type tumors (the tumor cells co-express bivalent differentiation and show neuroendocrine and exocrine features) [4,6,17–19].

As the poorly differentiated colorectal adenocarcinomas may express the neuroendocrine markers [20], the denomination of adenocarcinoma with neuroendocrine component is recommended for carcinomas showing neuroendocrine component in few than 30% of the tumor cells. However, this assessment is difficult to be performed by a non-experienced pathologist. Based on this fact, we have previously hypothesized that MANEC is rather a poorly differentiated adenocarcinoma with neuroendocrine component and should be treated using the guidelines for adenocarcinomas and not for neuroendocrine tumors [7]. Based on the positivity of CD44 in both components, we still sustain that the histogenesis of MANEC is based on a common precursor cell, for both adenocarcinoma and neuroendocrine tumor cells. The hypothesis of monoclonal origin of the two components was also highlighted by other authors, based on the positivity for other biomarkers of cancer stem cells such CD133 [7] and prominin 1 [4], and similar gene profile of microdissected cells from MANEC (both components), NEC and adenocarcinomas [2,4,21,22]. Compared to pure adenocarcinomas, *BRAF* and *p53* mutations seems to be more frequent in MANECs and K-ras, APC and other genes such as ATM, CTNNB1, ERBB4, JAK3, KDR and RB1are more rarely mutated [21,22]. We have

Table 1
The clinicopathological features of patients with gastrointestinal MANEC.

Case no	Gender	Age (years)	Tumor localization	Macroscopy	Microscopy – adenocarcinoma part	Microscopy – neuroendocine part	Lymph node Metastases (histology type)	pT stage	PN stage	Lymphatic invasion	Vascular invasion	Resection margins	Follow-up
1	F	74	Cecum	Polypoid-ulcerated	Low-grade G2 with mucinous component	Medium sized cells, acinar-trabecular, G1	–	4b	N0 (0/40)	L1	V1	R0	Died at 50 mo
2	F	78	Upper rectum	Ulcero-infiltrative	Low-grade G2	Medium sized cells, acinar-trabecular, G1	Neuroendocrine (4/4)	3	N1 (4/7)	L1	V1	R0	Died at 6 mo
3	F	63	Ascending colon	Polypoid	High-grade G2	Small cell, G3	Neuroendocrine (7/7)	4a	N2b (7/16)	L1 - emboli	V0	R1	Died at 13 months
4	M	47	Lower rectum	Ulcero-infiltrative	Low-grade G3	Large cell, trabecular, G3	Adenocarcinoma (1/7) and neuroendocrine (6/7)	4a	N2b (7/28)	L1 - emboli	V0	R1	Died at 10 months
5	F	54	Upper rectum	Polypoid	Low-grade G2	Medium sized cells, acinar-trabecular, G2	Neuroendocrine (1/1)	1a	N1 (1/9)	L1	V0	R0	Alive – 20 mo
6	M	72	Cecum	Polpypoid-ulcerated	High grade G3	Medium sized cells, acinar-trabecular, G3	Adenocarcinoma (1/3) and neuroendocrine (2/3)	4b	N1b (3/4)	L1 – emboli	V1	R0	Died at 7 months
7	M	70	Upper rectum	Polpypoid-ulcerated	Mucinous	Large cells clusters	–	3	N0 (0/12)	L1	V0	R0	NA
8	M	68	Gastroesophageal junction	Ulcero-infiltrative	Low-grade G2 with mucinous component (10%)	Medium sized cells, acinar-trabecular, G2	–	3	N0 (0/29)	L1	V0	R0	Alive – 32 mo
9	M	69	Cardia	Polypoid-ulcerated	Low-grade G2	Medium sized cells, acinar-trabecular, G3	Adenocarcinoma (1/2) and neuroendocrine (1/2)	2	N1 (2/18)	L1	V0	R0	Died at 12 months
10	M	65	Proximal stomach	Polypoid	Low-grade G2 with mucinous component	Large cells, trabecular, G3	–	2	N0 (0/27)	L1 – emboli	V1 – emboli	R0	Alive – 38 mo
11	M	70	Samll curvature – proximal stomach	Ulcero-infiltrative	Low-grade G2	Large cells, trabecular, G2	Adenocarcinoma (2/5) and neuroendocrine (3/5)	3	N1 (5/61)	L1	V0	R0	Died at 2 mo
12	M	46	Antrum	Polypoid	High-grade G2	Large cells, clusters – G3	Adenocarcinoma (5/22) and neuroendocrine (17/22)	4b	N3b (22/32)	L1	V1	R0	Died at 10 mo
13	F	63	Antrum	Ulcero-infiltrative	High-grade G2	Medium sized cells, trabecular	Neuroendocrine (2/2)	2	N1 (2/15)	L1	V0	R0	Died at 8 mo

Table 2
The immunohistochemical markers used to explore the tumor microenvironment.

Antibody (company)	Clone	Dilution
Synaptophysin (Dako)	DAK-Synap	RTU
Chromogranin (Dako)	DAK A3	RTU
NSE (Dako)	M0873	1:100
Keratin AE1/AE3 (Dako)	AE1/AE3	1:100
Keratin 7 (Thermo Scientific)	OV-TL 12/30	1:100
Keratin 20 (Thermo Scientific)	Q6	1:100
CEA (Thermo Scientific)	Ab3	1:200
S100 (Thermo Scientific)	Polyclonal	1:4000
MLH-1 (Leica)	ESO5	1:50
MSH-2 (Leica)	25D12	1:50
MSH-6 (Leica)	PU29	1:100
PMS-2 (Leica)	M0R4G	1:50
Maspin (Novocastra)	EAW24	1:50
E-cadherin (Dako)	NCH-38	1:50
β-catenin (Leica)	17 C2	1:50
N-cadherin (Dako)	6G11	1:100
Vimentin (Dako)	V9	1:800
CD44 (Leica)	DF1485	1:50
Ki67 (Dako)	MIB1	RTU

performed gene analysis in one of the cases (case 6) and identified a *K-ras* mutation (codon 61).

Poor response to the used regimens for NEC, followed by good answer at the adenocarcinoma regimens, even in metastatic cases (e.g. anti-EGFR drugs for K-ras wild type tumors – cetuximab/irinotecan regimen) was reported in some papers [23–25]. In other papers, 5-Fluorouracil or oxaliplatin-based multidrug chemotherapy was recommended for gastroenteropancreatic MANECs, as for adenocarcinomas, with a response rate of 40 % [10,14,16]. In our material, only four of the cases displayed pure neuroendocrine lymph node metastases and might respond to NEC-related chemotherapic regimens. However, MANECs are still treated using the guidelines for NEC and not for adenocarcinomas.

The guidelines of the European Society of Medical Oncology (ESMO) recommend platinum-based therapy for metastatic cases (not include the cases with metastases in the regional lymph nodes) and everolimus or amrubicin in patients with platinum-refractory metastatic or inoperable MANECs/NECs of the gastrointestinal tract [24]. The reported response rate at amrubicin is below 20 %, with a median progression-free survival of about 3–4 months and median overall survival below 8 months, with a grade 3/4 neutropenia occurring in 52.6 % of patients [24]. As in our material the lymph node metastases proved to influence the evolution, we consider that the locally advanced MANECs should also benefit from chemotherapy. The National Comprehensive Cancer Network Guidelines (version 1.0 2019) recommend to treat gastrointestinal tumors with mixed histology based on adenocarcinoma guideline and not neuroendocrine tumors guideline [26]. In two of the case reports published in 2019, it was also suggested that immunotherapy with Pembrolizumab might be used in both gastric and colorectal PDL-1 positive MANECs with liver metastases [27,28].

MANEC's proved to have aggressive behavior with high risk of local and distant metastases, similar to our material [9,16]. In the previously studies, it was shown that about 75% of the patients present lymph node metastases and distant metastases in liver or bone were identified in 20 % of the colorectal MANECs [5]. It was hypothesized that the overall survival is longer in patients with MANEC, compared with pure NEC [17] and lower, compared with pure adenocarcinomas [14,16], but other authors denied this aspect [5,21]. Moreover, most of the NECs have a well-defined glandular component, sometimes with mucinous content, although it is lower than 30 % [2]. Due to this fact, it is still unclear whether the adenocarcinoma component or the neuroendocrine component is the more aggressive part responsible for the disease progression and metastases [7,19,29,30].

Although it was postulated that gastric MANEC is less aggressive than those located in the colorectal segments are, the site of the tumor is not yet included in the prognostic factors of these tumors [25]. The median overall survival is about 12–25 months for stomach, 17–18 months for colorectal and 15 months for ampullary MANECs [6,10,14,16,30]. The follow-up of patients with gastric MANEC/NEC can be performed using the (18)F-fluorodeoxyglucose positron emission tomography [30].

More than 44 % of patients with colorectal MANECs were reported to succumb to the disease in first year after diagnosis, one third of them in the first 5.5 months [5,16]. The median progression-free survival rate

Table 3
The immunoprofile of the two components of gastrointestinal MANEC.

BIOMARKER	GASTRIC MANEC (n = 6)		COLORECTAL MANEC (n = 7)	
	Neuroendocrine component	Adenocarcinoma component	Neuroendocrine component	Adenocarcinoma component
Synaptophysin	Diffuse positivity (6/6)	Negative (5/6) or focal positivity (1/6)	Diffuse (6/7) or focal positivity (1/7)	Negative (6/7) or focal positivity (1/7)
Chromogranin	Negative (2/6) or positive (4/6)	Negative (4/6) or positive (2/6)	Negative (2/7) or positive (5/7)	Negative (7/7)
NSE	Diffuse (3/6) or focal positivity (3/6)	Negative (6/6)	Diffuse positivity (7/7)	Negative (6/7) or focal positivity (1/7)
Keratin AE1/AE3	Negative (4/6) or positive (2/6)	Diffuse positivity (6/6)	Negative (5/7) or positive (2/7)	Diffuse positivity (7/7)
Keratin 7	Negative (6/6)	Focal positivity (3/6) or negative (3/6)	Negative (7/7)	Negative (7/7)
Keratin 20	Negative (6/6)	Focal positivity (2/6) or negative (4/6)	Negative (4/7) or focal positivity (3/7)	Diffuse (4/7) or focal positivity (3/7)
CEA	Negative (3/6) or focal positivity (3/6)	Focal positivity (6/6)	Negative (5/7) or focal positivity (2/7)	Diffuse positivity (7/7)
S100	Negative (6/6)	Negative (5/6) or cytoplasmic positivity (1/6)	Negative (7/7)	Negative (7/7)
MLH-1	Positive (6/6)	Positive (6/6)	Positive (7/7)	Positive (7/7)
MSH-2	Positive (6/6)	Positive (6/6)	Positive (7/7)	Positive (7/7)
MSH-6	Positive (6/6)	Positive (6/6)	Positive (7/7)	Positive (7/7)
PMS-2	Positive (6/6)	Positive (6/6)	Positive (7/7)	Positive (7/7)
Maspin	Cytoplasm only (4/6) or negative (2/6)	Mixed expression – cytoplasm and nucleus (6/6)	Negative (7/7)	Negative (7/7)
E-cadherin	Diffuse positivity (6/6)	Diffuse positivity (6/6))	Diffuse positivity (7/7)	Diffuse positivity (7/7)
β-catenin	Membrane positivity (6/6)	Membrane positivity (6/6)	Membrane positivity (7/7)	Membrane positivity (7/7)
N-cadherin	Negative (6/6)	Negative (6/6)	Negative (7/7)	Negative (7/7)
Vimentin	Negative (6/6)	Negative (6/6)	Negative (7/7)	Negative (7/7)
CD44	Membrane positivity (6/6)	Membrane positivity (6/6)	Membrane positivity (7/7)	Membrane positivity (7/7)

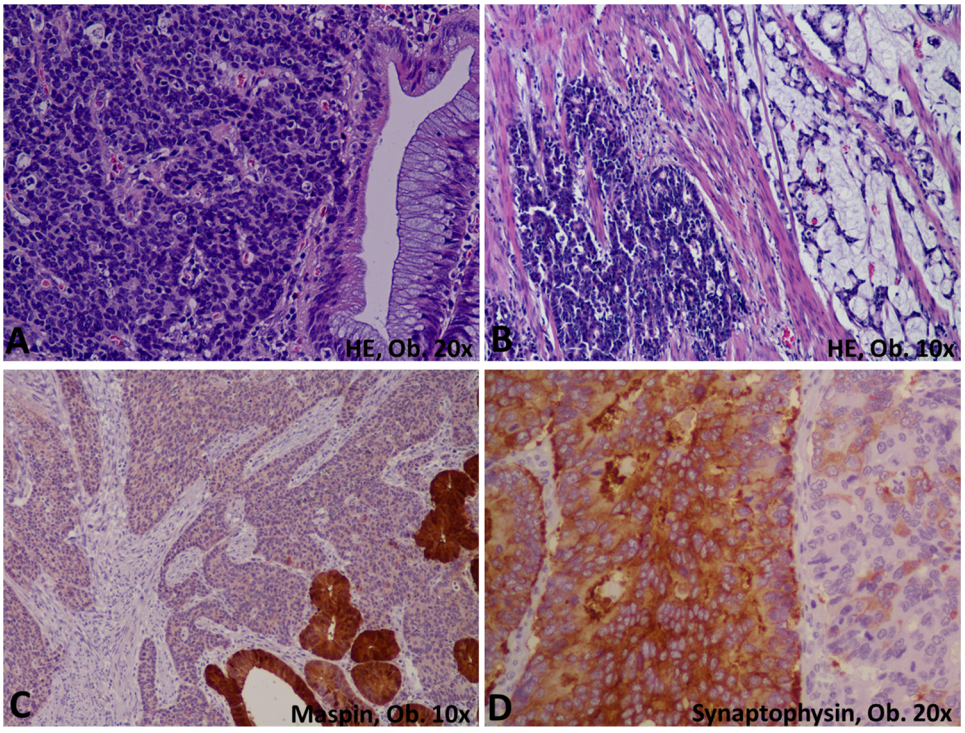

Fig. 1. In gastric MANEC without (A) or with mucinous component (B), the neuroendocrine component is negative for Maspin (C) and positive for synaptophysin (D).

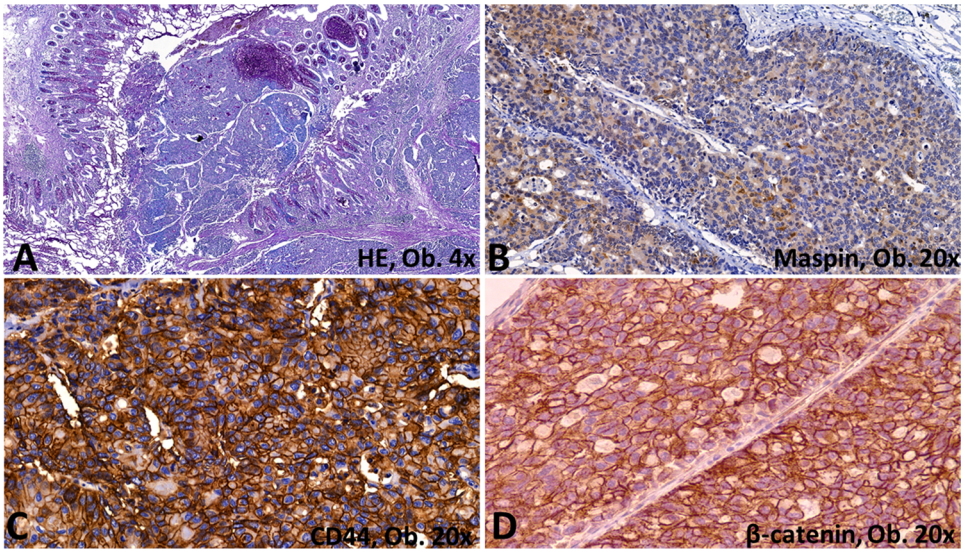

Fig. 2. In a MANEC of the cecum (A–D), the tumor cells from both components are negative for maspin (B) and diffusely membrane expression can be seen for CD44 (C) and β-catenin (D).

is about 6 months and mainly depends on the tumor stage [14]. In patients with colorectal MANECs, the 5-year disease free survival is 100 % for first stage, 73 % for stage II and 44 % for stage III [5,14,16]. In patients with gastric MANEC, lymph node metastases and tumor relapse status were proved as the main prognostic factors, similar to our study [10,30]. The evolution was influenced by the histology of the lymph node metastatic cells [30]. It was postulated that the stemness features, proved with CD44 in the present study, induces aggressively behaviour and might explain the high risk for lymphatic invasion [13,31].

In our study we also showed first time in literature that E-cadherin and β-catenin are diffusely expressed in the membrane of the tumor cells, without β-catenin nuclear expression, without N-cadherin, S100 or vimentin positivity. This proves that the EMT of the tumor cells does not induce the MANEC aggressivity. Our data are in agreement with two of the recent studies showing diffuse E-cadherin positivity [11] and only rare *CTNNB1* gene mutations in patients with MANEC [22]. β-catenin nuclear expression is an indicator of gene mutation and loss of cell adhesivity in the invasion front, but was not identified in the present material.

Maspin negativity or cytoplasmic only expression, without nuclear expression, and without tumor buds in the neuroendocrine component [12] also indicate that particular pathways are involved in these tumors. The budding quantification [12,13] was not helpful in any of the cases; the neuroendocrine components did not displayed stromal isolated cells.

Limitations of the study consist in one-center used database, retrospective nature and limited number of cases. However, most of the previously published paper about MANEC were case reports. The

limitations were compensated by using a large immunophenotype, which was not previously reported in other publications.

In conclusion, this study shows that the EMT and maspin cannot be defined as prognostic factors, in patients with gastric or colorectal MANECs. MANEC is a microsatellite stable tumor in which the tumor buddings have not a prognostic value. CD44 can influence MANEC histogenesis but the evolution mainly depends on the lymph node status. For this reason, therapeutical management should be oriented on the predominant component (adeno- versus neuroendocrine), not only in the primary tumor but also in the lymph node metastases.

Author contributions

Concept – GS, JI, FA; Design – JI, BL, TV; Supervision – JI; Funding – GS, BT; Materials – BTJ, BL; Data Collection and/or Processing – SM, TV, BVO; Analysis and/or Interpretation – SM, BL, FA; Literature Review – TV, FA, SM, BVO; Writing Manuscript – GS, BVO; Critical Review – BT, JI, TV; Surgical interventions – BT, BTJ.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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